Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 8103–8107, October 1989
Neurobiology

Discrimination of heterogenous mRNAs encoding strychnine-sensitive glycine receptors in *Xenopus* oocytes by antisense oligonucleotides

(spinal cord/hybrid-arrested translation/synthetic DNA/blot hybridization/development)

HIROYUKI AKAGI*, DAVID E. PATTON, AND RICARDO MILEDI†

Laboratory of Cellular and Molecular Neurobiology, Department of Psychobiology, University of California, Irvine, CA 92717

Contributed by Ricardo Miledi, July 3, 1989

ABSTRACT **Three synthetic oligodeoxynucleotides complementary to different parts of an RNA encoding a glycine receptor subunit were used to discriminate heterogenous mRNAs coding for glycine receptors in adult and neonatal rat spinal cord. Injection of the three antisense oligonucleotides into *Xenopus* oocytes specifically inhibited the expression of glycine receptors by adult spinal cord mRNA. In contrast, the antisense oligonucleotides were much less potent in inhibiting the expression of glycine receptors encoded by neonatal spinal cord mRNA. Northern blot analysis revealed that the oligonucleotides hybridized mostly to an adult cord transcript of ≈10 kilobases in size. This band was also present in neonatal spinal cord mRNA but its density was about one-fourth of the adult cord message. There was no intense band in the low molecular weight position (≈2 kilobases), the existence of which was expected from electrophysiological studies with size-fractionated mRNA of neonatal spinal cord. Our results suggest that in the rat spinal cord there are at least three different types of mRNAs encoding functional strychnine-sensitive glycine receptors.**

Glycine is a major inhibitory neurotransmitter in the mammalian central nervous system and is predominant in the spinal cord. Neuronal inhibition induced by glycine arises from the opening of chloride channels operated by glycine receptors, which can be blocked by a selective antagonist, strychnine (1, 2).

Many attempts at elucidating the molecular structure of glycine receptors are in progress (3). We (4–11) and others (12–17) have shown that mRNAs extracted from the brain and spinal cord of various animals, including human, induce *Xenopus* oocytes to express functional voltage-dependent ionic channels and neurotransmitter receptors, including strychnine-sensitive glycine receptors. We have recently shown that the glycine receptor mRNA derived from adult rat spinal cord differed from that of adult rat cerebral cortex in molecular size and that some of the properties of the receptors encoded were also different (18). Moreover, the characteristics of the glycine receptor mRNA were shown to change with postnatal development of the spinal cord (18). Thus, the glycine receptor and its mRNA are not homogenous, but rather it appears that multiple glycine receptor mRNAs exist in the central nervous system.

Recently, a cDNA clone, encoding the strychnine binding subunit of the glycine receptor from the spinal cord of 20-day-old rats, was isolated, sequenced, and expressed (19, 20). The information derived from that sequence is very useful for distinguishing heterogenous glycine receptor mRNAs. Based on that sequence we prepared three synthetic oligodeoxynucleotides complementary to the mRNA coding for the strychnine binding protein. These antisense nucleotides were used to determine their ability to repress (see refs. 19 and 21) the synthesis of functional glycine receptors in *Xenopus* oocytes injected with native mRNA, and radiolabeled probes were used to detect glycine receptor mRNAs.

MATERIALS AND METHODS

RNA Preparation. Poly(A)$^+$ mRNAs were extracted from the spinal cord of adult and neonatal (3–4 days old) rats and purified by oligo(dT)-cellulose chromatography as described (11, 18). Part of the mRNAs was fractionated by linear sucrose (32% to 10%; wt/vol) density gradient centrifugation (260,000 × *g* for 13 hr at 4°C) in an SW 41 rotor (Beckman), and 400-μl fractions were collected, precipitated with ethanol, dried, and then suspended in 20 μl of water (18, 22).

Antisense Oligonucleotides. Three 30-mer oligodeoxynucleotides complementary to the mRNA coding for a strychnine binding subunit of the glycine receptor (19) were synthesized by F. Hishinuma in an automatic DNA synthesizer. The sequence of the antisense oligonucleotides and their corresponding regions in the cDNA are shown in Table 1. The antisense nucleotides were desalted and partially purified by Sephadex G-25 column chromatography, and purity was checked by polyacrylamide gel electrophoresis and staining with ethidium bromide. The purified antisense nucleotides were dissolved in water to make a 100 μM stock solution and stored at −70°C. The concentration was determined by measuring the optical density at 260 nm (22).

***In Vivo* Translation and Electrophysiological Recordings.** The solutions containing total mRNAs (2 μg/μl) or fractionated mRNAs were mixed with an equal volume of water (as control) or of a solution containing an antisense nucleotide (100 fmol to 10 pmol/μl), and 50 nl of the mixture was injected into *Xenopus* oocytes. On the second or third day after injection, the oocytes were treated with collagenase (Sigma type I; 0.5–1 mg/ml) for about 1 hr to remove follicular and other enveloping cells. From the fourth to seventh days after injection, the activities of the receptors expressed were examined electrophysiologically with the oocyte membrane under voltage-clamp (23).

Blot Hybridization. mRNAs (20 μg of each preparation) were electrophoresed in a 1% (wt/vol) agarose gel containing 0.66 M formamide, blotted onto a hybridization transfer membrane (GeneScreen*Plus*; DuPont), and fixed by UV illumination. The oligonucleotides were 5′-end-labeled with [γ-^{32}P]ATP (Amersham) using T4 polynucleotide kinase (Pharmacia). The specific activity of the probes was about 6

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: AS, antisense; GABA, γ-aminobutyric acid; 5-HT, 5-hydroxytryptamine.

*Present address: Department of Neuroscience, Mitsubishi Kasei Institute of Life Sciences, Machida-shi, Tokyo 194, Japan.
†To whom reprint requests should be addressed.

Table 1. Oligodeoxynucleotide sequences

Antisense code	5′ → 3′ sequence	Corresponding base number of the cDNA*	% (G+C)
AS-I	GGG TGA CAT AGG CTT GGG TGC AGA GCG GGC	21–50	66
AS-II	TGC CAC CTG CAC AGC TCC TTG CTC TTG CCA	527–556	60
AS-III	TTT GTC GAT CTT CTT GGC TCT CTG GAT GAA	1158–1187	43

*See ref. 19.

$\times 10^8$ cpm/μg. Hybridization was carried out for 15 hr at 42°C and the membrane was washed in 0.3 M NaCl/0.03 M sodium citrate buffer containing 1% SDS at 70°C (24). Autoradiographs were made on x-ray film (RX; Fuji) at −70°C.

RESULTS

Selective Repression of Glycine Receptors by Antisense Nucleotides. As previously reported (11), injection of mRNAs from adult rat spinal cord into oocytes induced the expression of glycine receptors that generate Cl^- currents in response to glycine (Fig. 1). In contrast to oocytes injected with adult brain mRNAs, in which a relatively small number of glycine receptors are expressed, the amplitude of the glycine-induced response in oocytes injected with spinal cord mRNA was usually the largest among the currents elicited by amino acid receptor agonists, such as γ-aminobutyric acid (GABA), β-alanine, and kainate (compare Fig. 2*A*; see also ref. 11). In the oocytes coinjected with spinal cord mRNA and the 5′ oligonucleotide, AS-I, the number of glycine receptors expressed, and therefore the amplitude of the glycine-induced current, was much smaller than that in control oocytes and depended on the amount of AS-I injected. As shown in Fig. 1, injecting 2.5 fmol of AS-I had little or no effect on the amplitude of the elicitable glycine currents, whereas injecting 42 fmol almost completely suppressed the expression of glycine receptors and the current elicited by glycine was only 1–5% of the control value.

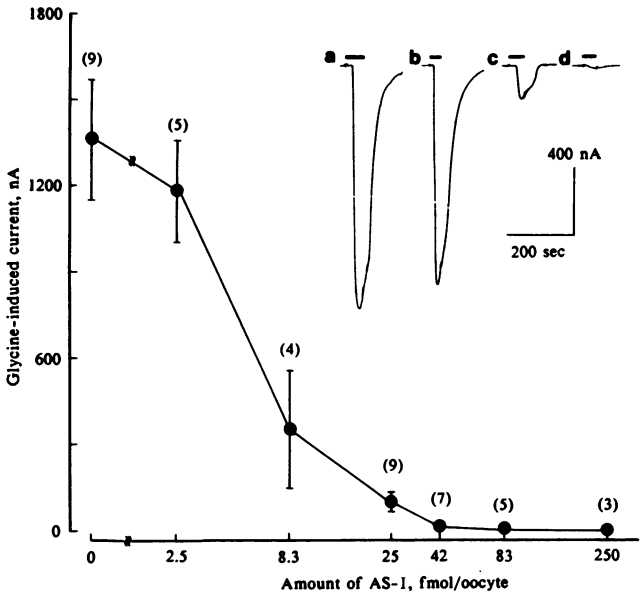

FIG. 1. Glycine-induced membrane currents in *Xenopus* oocytes injected with adult rat spinal cord mRNA alone or with an antisense oligonucleotide, AS-I, at various concentrations. The oocytes were voltage-clamped at −60 mV and perfused with 1 mM glycine. Each point represents the peak amplitude of the glycine currents (±SEM) obtained in each group of oocytes (number in parentheses). (*Inset*) Sample records of glycine currents in oocytes injected with AS-I at 0 (a), 2.5 (b), 25 (c), and 83 fmol (d). Glycine was added to the bathing solution during the periods indicated by horizontal bars. Downward deflection denotes inward current. All oocytes derived from a single donor.

To see if the action of AS-I was specific for glycine receptors the effect of coinjecting spinal cord mRNA and AS-I on the expression of other receptors and ion channels was examined. The AS-I (42 fmol) did not affect the amplitudes of voltage-dependent currents such as tetrodotoxin-sensitive Na^+ currents (Fig. 2*B*) and Ca^{2+}-dependent Cl^- currents (due to expression of calcium channels) or the sizes of the responses elicited by GABA (1 mM), kainate (0.1 mM), and 5-HT (10 μM) acting on their respective receptors (Fig. 2). As previously shown (11, 18), oocytes injected with adult cord mRNAs elicit large inward currents in response to β-alanine. These β-alanine currents were also greatly depressed in the oocytes coinjected with AS-I (Fig. 2). Thus, the AS-I at 42 fmol exhibited considerable specificity in repressing the expression of receptors activated by glycine and β-alanine.

Effects of Other Antisense Nucleotides on the Expression of Glycine Receptors Encoded by Adult Cord mRNA. The repressional potency of the other two antisense nucleotides, AS-II and AS-III, on glycine currents in oocytes injected with adult cord mRNAs was compared to that of AS-I. As shown in Fig. 3*A*, coinjection with AS-III caused a marked suppression of the glycine currents, similar to that caused by AS-I. In contrast, the repressional effect of AS-II (42 fmol) was considerably weaker and the glycine current was reduced only to 57% of the control response (Fig. 3*A*). Furthermore, even at a higher concentration (250 fmol), AS-II did not suppress the expression of glycine receptors as much as the lower concentration of AS-I (Fig. 3*A*).

Effects of the Antisense Nucleotides on the Expression of Glycine Receptors Encoded by mRNA from Neonatal Rat Spinal Cord. Oocytes injected with mRNA derived from 3- to 4-day-old rat spinal cords elicited inward currents whose amplitude was comparable to that of oocytes injected with mRNA from adult spinal cord (11). Moreover, as in oocytes injected with adult cord mRNAs, the glycine current was mainly due to an increase in Cl^- permeability and was blocked by strychnine (11). Thus, the glycine receptors encoded by neonatal cord mRNAs share some of the properties of receptors encoded by adult cord mRNA. Nevertheless, the potency of the antisense nucleotides in repressing the synthesis of neonatal cord glycine receptors in the oocytes was markedly different from that exerted on the synthesis of glycine receptors encoded by adult spinal cord mRNA. For instance, with 42 fmol, none of the three antisense nucleotides significantly reduced the amplitude of the glycine currents in oocytes injected with neonatal cord mRNAs. Moreover, even after injection of a higher amount of antisense nucleotide (250 fmol), the average amplitude of the glycine-induced current was reduced to only 63–73% of the control response (Fig. 3*B*).

Effect of Antisense Nucleotides on the Expression of Glycine Receptors Encoded by Fractioned mRNAs of Adult and Neonatal Spinal Cord. The results mentioned above (see also ref. 18) suggested the existence of heterogenous mRNAs coding for strychnine-sensitive glycine receptors in the rat spinal cord. This possibility was investigated further in oocytes injected with partially purified mRNAs obtained by sucrose

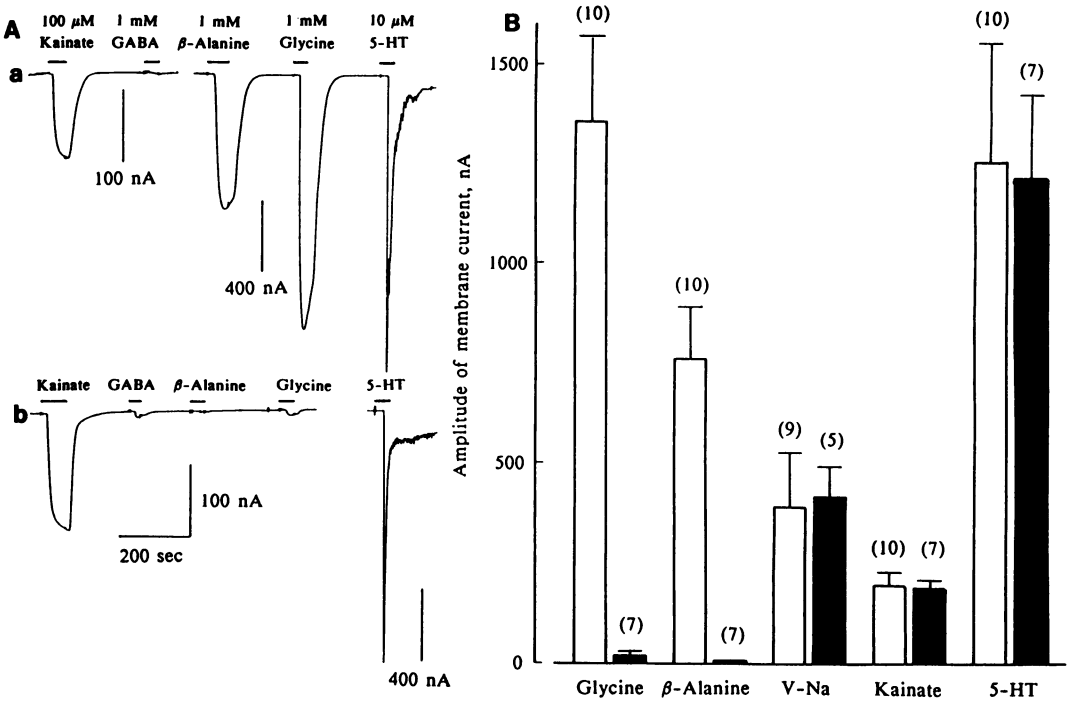

FIG. 2. Effect of AS-I on various types of membrane currents elicited in oocytes injected with adult spinal cord mRNA. Oocytes were injected with 50 nl of mRNA (2 μg/μl) mixed with an equal amount of AS-I (1.68 pmol/μl). (*A*) Sample records of neurotransmitter responses in oocytes clamped at −60 mV. Agonists were applied as indicated by the bars. a, Responses in a control oocyte; b, same sequence of responses in an oocyte coinjected with AS-I (42 fmol). 5-HT, 5-hydroxytryptamine (serotonin). (*B*) Peak amplitude of inward currents activated by voltage-dependent Na^+ channels (V-Na) and neurotransmitter receptors in control oocytes (open columns) or oocytes injected with AS-I (solid columns). The Na^+ currents were measured by stepping the clamp potential to −20 mV from a holding potential of −100 mV. Each column represents the average value (with SEM) obtained in oocytes (number in parentheses) derived from a single donor.

density gradient centrifugation and subsequent fractionation. Fig. 4 shows the distribution of adult spinal cord mRNAs coding for glycine receptors in the sucrose gradient. In accord with our previous results (18), most of the adult cord mRNA expressing glycine receptors sediments faster than the 28S rRNA. This profile is the opposite of that obtained with neonatal cord mRNA, in which most of the mRNA expressing glycine receptors sediments close to the 18S rRNA (see figure 1C in ref. 18).

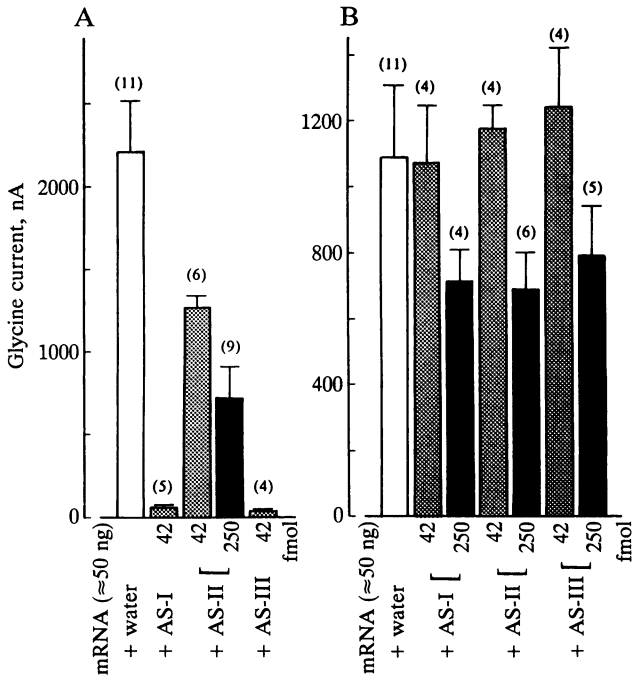

FIG. 3. Effect of antisense oligonucleotides on glycine receptor synthesis in oocytes injected with adult (*A*) or neonatal (*B*) rat spinal cord mRNA, with or without the oligonucleotides. Each column represents the mean amplitude (with SEM) of the currents induced by 1 mM glycine.

The effect of the antisense nucleotides on the expression of glycine receptors from fractionated spinal cord mRNA is shown in Fig. 5. All three antisense nucleotides (42 fmol) markedly suppressed the synthesis of glycine receptors derived from adult cord mRNA fraction 8 (Fig. 5*A*). In contrast, AS-II was relatively ineffective in repressing the expression of receptors by fraction 9 (Fig. 5*B*).

The expression of glycine receptors by neonatal cord mRNA fraction 13 (for profile, see ref. 18) was barely altered by coinjection of any of the three antisense nucleotides, even at 250 fmol (Fig. 5*C*), in contrast to the ≈30% repression seen with unfractionated mRNA (compare Fig. 3*B*).

Antisense Nucleotides Hybridize to Large Transcripts in Spinal Cord mRNA. A preliminary attempt was made to discriminate heterogenous spinal cord mRNAs coding for glycine receptors by blot hybridization using the radiolabeled antisense oligonucleotides as probes. In agreement with previous results, obtained with a much larger probe (19), the smaller AS-I again recognized mostly transcripts of large molecular size (≈10 kb, band 1 in Fig. 6) in the adult cord mRNA. A similarly intense band was not been with poly(A)$^-$ RNA or with adult cerebral cortex mRNA, suggesting that the large mRNA is fairly specific for the spinal cord. In addition, the adult spinal cord mRNA gave less intense bands in positions close to ≈5, ≈3, and ≈2 kb (bands 2–4). The band at ≈10 kb was also found in the neonatal spinal cord mRNA, although its intensity was about four times less than that of the adult cord transcript. However, AS-I did not disclose a strong band near the 2-kb range, as would be expected from

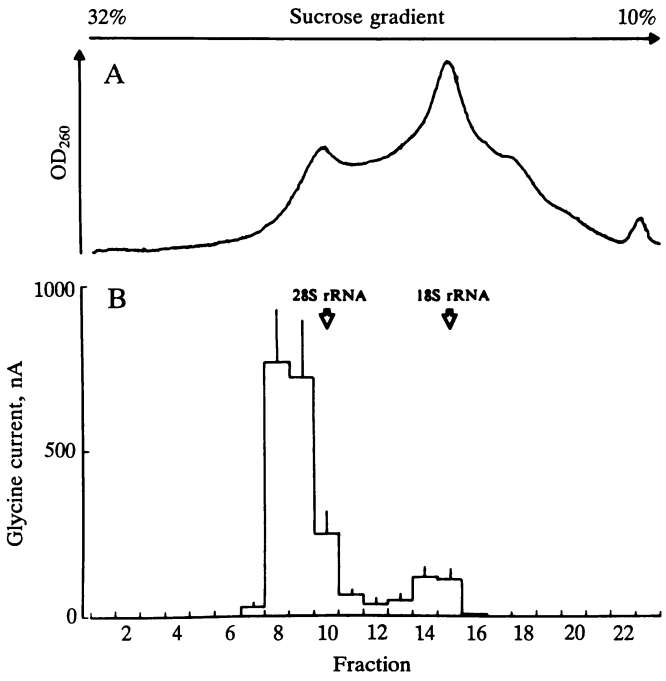

FIG. 4. Distribution of adult rat spinal cord mRNA encoding glycine receptors after fractionation by sucrose gradient centrifugation. (*A*) Optical density at 260 nm monitored continuously while fractionating the sucrose gradient solution. (*B*) The fractionated mRNAs were suspended in 20 μl of water. Aliquots of each fraction were further diluted three times and 50 nl of this was injected into oocytes. The columns represent the mean amplitude of the response to 1 mM glycine in three to eight oocytes. Bars indicate SEM.

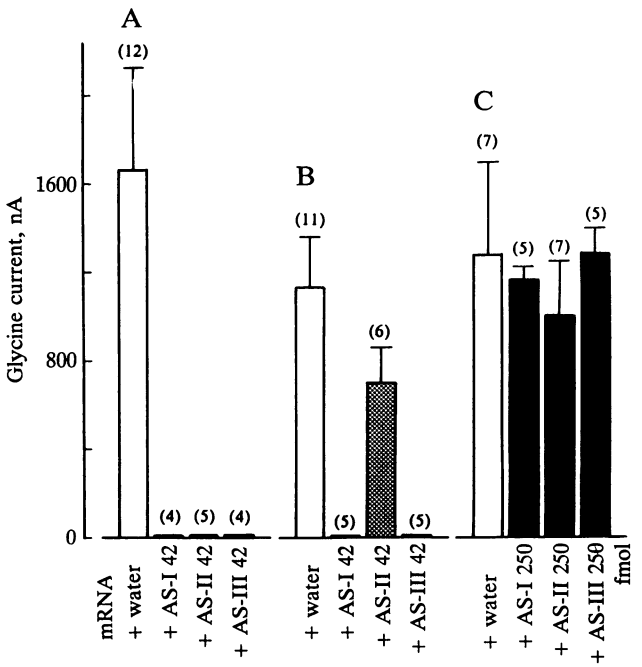

FIG. 5. Repressional effects of the oligonucleotides on glycine receptor expression in oocytes injected with adult rat spinal cord mRNA fractions 8 (*A*) and 9 (*B*) and neonatal spinal cord fraction 13 (*C*). For the sedimentation profile of the neonatal spinal cord mRNA, see ref. 18. Each mRNA fraction was mixed with an equal volume of water, or the solution containing the oligonucleotides, and the mixture was injected into oocytes. Columns represent the mean amplitude (with SEM) of the glycine current in oocytes (number in parentheses) derived from two different donors.

the fact that neonatal spinal cord mRNA of that size was able to express a large number of functional glycine receptors (18). Autoradiographs with AS-II and AS-III gave essentially similar results as with AS-I (data not shown).

DISCUSSION

Evidence has been accumulating that in the central nervous system, mRNAs coding for voltage- or transmitter-operated ion channels exist in heterogenous forms. For instance, the α subunit of tetrodotoxin-sensitive Na^+ channels can be translated from four different types of mRNAs (25–27). Other cases have been reported for potassium channels (28), nicotinic acetylcholine receptors (29, 30), and GABA receptors (31). The strychnine-sensitive glycine receptor may also be synthesized from different species of mRNA, and evidence showing this has already been given (18). In the present study, synthetic oligonucleotides complementary to the mRNA coding for the glycine receptor of 20-day-old rat spinal cord were used to examine further the heterogeneity of the glycine receptor mRNAs.

It is well known that DNA, RNA, and oligonucleotides complementary to a target mRNA block the synthesis of specific proteins *in vitro* or *in vivo* (21, 32–36). The mechanism of this repression appears to be complex (36), but an important step in the repression process is initial hybridization of the probe to the target mRNA. We have shown that the expression of functional glycine receptors in oocytes was inhibited by coinjecting the spinal cord mRNA with small amounts (20–250 fmol) of antisense oligonucleotides and shown also that the 5′- and 3′-directed antisense RNAs were similarly effective. Assuming that the content of mRNAs coding for glycine receptors in the adult spinal cord mRNAs is 0.1%, and the molecular weight is 3.5×10^6 (corresponding to 10 kb in length), the amount of glycine receptor mRNAs injected into the oocytes would be 0.02 fmol, an amount 1000–10,000 times smaller than the amount of antisense required for effective repression. With longer antisense RNAs effective inhibition has been obtained even with nearly equimolar ratios (21). The estimate given above also suggests that injection of the cloned 48-kDa subunit RNA alone (20) is much less efficient in expressing glycine receptors than native mRNA.

The repressional effect of the antisense nucleotides on protein synthesis in oocytes was specific to glycine receptors: the synthesis of other types of neurotransmitter receptors and voltage-operated channels was not affected by antisense

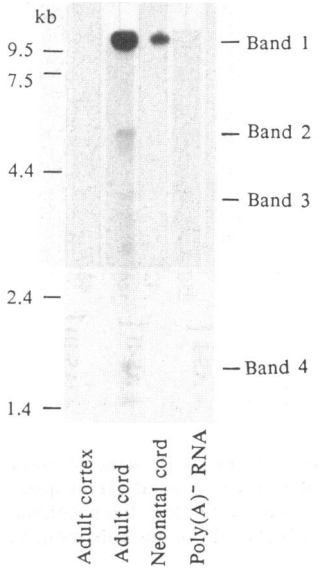

FIG. 6. Blot hybridization analysis with AS-I and mRNAs from adult rat cerebral cortex (lane 1) and adult and neonatal rat spinal cords (lanes 2 and 3) after gel electrophoresis. Poly-$(A)^-$ RNA (lane 4) was obtained from adult rat spinal cord (the break-through fraction on oligo(dT)-cellulose). An RNA ladder (BRL) was used for size markers.

nucleotide concentrations up to 250 fmol, although higher concentrations had nonspecific effects. β-Alanine is an amino acid structurally similar to glycine and GABA. It produces inhibition in neuronal cells (1) but its mode of action is still unclear. It has been postulated that glycine, GABA, and/or specific β-alanine receptors are involved in the inhibition by this amino acid in the nervous system (1, 9). Our finding that the antisense nucleotides caused the suppression of both glycine- and β-alanine induced currents suggests that, at least in adult rat spinal cord, the action of this amino acid is mainly elicited by glycine receptors, or a very homologous specific β-alanine receptor.

We have shown previously that most of the glycine receptor mRNA in the neonatal rat spinal cord differs from that in the adult spinal cord (11, 18). For instance, after sucrose density gradient centrifugation of neonatal cord mRNA most of the mRNA that expresses glycine receptors in oocytes sediments in a low density position (close to 18S rRNA), whereas in adult spinal cord mRNA, most of the glycine receptor mRNA is heavier than 28S rRNA (see Fig. 4 and also ref. 18). Furthermore, some electrophysiological properties of the glycine receptors encoded by neonatal cord mRNA differed from those of receptors encoded by adult cord mRNAs (18). In the present study, we found that AS-I and AS-III suppressed almost completely the expression of glycine receptors encoded by adult cord mRNA but inhibited by ≈30% or not at all the synthesis of glycine receptors in oocytes injected with either total or fraction 13 mRNA, respectively, from neonatal spinal cord. Accordingly, Northern blot analyses in the present (Fig. 6) and previous studies (19) revealed that except for the ≈10-kb transcript in neonatal spinal cord mRNA, there were no other detectable bands. Thus, it appears that most of the neonatal cord mRNA encoding glycine receptors is structurally different from that in the adult cord mRNA, although both types of receptors still have some similar basic properties, such as strychnine sensitivity. In this context, Becker *et al.* (37) have recently shown that neonatal rat spinal cord glycine receptors differ antigenically from those in the adult.

Another interesting outcome of the present study concerns the different potency of the oligonucleotides in inhibiting the expression of glycine receptors by the adult spinal cord mRNA. AS-I and AS-III markedly suppressed the synthesis of glycine receptors, whereas AS-II was considerably less potent at the same or even higher concentrations. This result is of great interest because all of the antisense nucleotides were complementary to the protein coding region of the 48-kDa subunit cDNA. It is known that the number of guanine and cytosine bases [(G+C) contents] in the probe affect the efficacy of hybridization of the probe to RNA (38). However, this does not seem to account for our results because the (G+C) content in AS-II is higher than in AS-III (Table 1), which produced marked repression (Fig. 3). Another possibility is that the secondary structure of the glycine receptor mRNA hinders the hybridization of AS-II. This possibility also seems unlikely because the AS-II was very potent in inhibiting the expression of glycine receptors by adult cord mRNA fraction 8. In contrast, the repressional potency of the AS-II for mRNA fractions 9 (Fig. 5) and 10 (data not shown), both of which contained high molecular weight (>5 kb) mRNAs (judging from the position of 28S rRNA), was similar to that for total mRNA. Based on these results, it seems that in adult rat spinal cord, there is another glycine receptor mRNA that contains a nucleotide sequence complementary to both AS-I and AS-III but not to AS-II. If this were the case, then the oligonucleotide probes would be expected to discriminate the receptor mRNAs in Northern blots. However, so far, we have not seen any striking difference in the hybridization patterns obtained with AS-I and with AS-II. This problem will be resolved when full-length clones of cDNAs encoding multiple strychnine-sensitive glycine receptors become available.

We are grateful to Dr. F. Hishinuma (Mitsubishi Kasei Institute of Life Sciences) for providing synthetic oligonucleotides and Drs. M. Panicker and K. Sumikawa for helpful comments on the manuscript. This work was supported by Grant R01-NS23284 from the U.S. Public Health Service. H.A. and D.E.P. were supported in part by grants from Mitsubishi Kasei Institute of Life Sciences (to H.A.) and from the National Institute of Mental Health (MH 14599 to D.E.P.).

1. Young, A. B. & Macdonald, R. L. (1983) in *Handbook of the Spinal Cord*, ed. Davidoff, R. A. (Dekker, New York), Vol. 1, pp. 1–43.
2. Barker, J. L. (1985) in *Neurotransmitter Actions in the Vertebrate Nervous System*, eds. Rogawski, M. A. & Barker, J. L. (Plenum, New York), pp. 71–100.
3. Betz, H., Schmitt, B., Becker, C.-M., Grenningloh, G. & Rienitz, A. (1987) in *The Molecular Biology of Receptors*, ed. Strosberg, A. D. (Ellis, Chichester, U.K.), pp. 128–138.
4. Miledi, R. & Sumikawa, K. (1982) *Biomed. Res.* **3**, 390–399.
5. Miledi, R., Parker, I. & Sumikawa, K. (1982) *Proc. R. Soc. London Ser. B* **218**, 418–484.
6. Gundersen, C. B., Miledi, R. & Parker, I. (1984) *Proc. R. Soc. London Ser. B* **221**, 127–143.
7. Gundersen, C. B., Miledi, R. & Parker, I. (1984) *Proc. R. Soc. London Ser. B* **221**, 235–244.
8. Gundersen, C. B., Miledi, R. & Parker, I. (1984) *Nature (London)* **308**, 421–424.
9. Parker, I., Sumikawa, K. & Miledi, R. (1988) *Proc. R. Soc. London Ser. B* **233**, 201–216.
10. Carpenter, M. K., Parker, I. & Miledi, R. (1988) *Proc. R. Soc. London Ser. B* **234**, 159–170.
11. Akagi, H. & Miledi, R. (1988) *Neurosci. Lett.* **95**, 262–268.
12. Dascal, N., Snutch, T. P., Lubbert, H., Davidson, N. & Lester, H. A. (1986) *Science* **231**, 1147–1150.
13. Asanuma, A., Horikoshi, T., Yanagisawa, K., Anzai, K. & Goto, S. (1987) *Neurosci. Lett.* **76**, 87–90.
14. Harada, Y., Takahashi, T., Kuno, M., Nakayama, K., Masu, Y. & Nakanishi, S. (1987) *J. Neurosci.* **7**, 3265–3273.
15. Nomura, Y., Kaneko, S., Kato, K., Yamagishi, S. & Sugiyama, H. (1987) *Mol. Brain Res.* **2**, 113–123.
16. Sigel, E. (1987) *J. Physiol. (London)* **386**, 73–90.
17. Verdoorn, T. A., Kleckner, N. W. & Dingledine, R. (1987) *Science* **238**, 1114–1116.
18. Akagi, H. & Miledi, R. (1988) *Science* **242**, 270–273.
19. Grenningloh, G., Rienitz, A., Schmitt, B., Methfessel, C., Zensen, M., Beyreuther, K., Gundelfinger, E. D. & Betz, H. (1987) *Nature (London)* **328**, 215–220.
20. Schmieder, V., Grenningloh, G., Schofield, P. R. & Betz, H. (1988) *EMBO J.* **8**, 695–700.
21. Sumikawa, K. & Miledi, R. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 1302–1306.
22. Sumikawa, K., Parker, I. & Miledi, R. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 7994–7998.
23. Kusano, K., Miledi, R. & Stinnakre, J. (1982) *J. Physiol. (London)* **328**, 143–170.
24. Fourney, R. M., Miyakoshi, J., Day, R. S., III, & Paterson, M. C. (1988) *Focus* **10**, 5–7.
25. Noda, M., Ikeda, T., Kayano, T., Suzuki, H., Takeshima, H., Kurasaki, M., Takahashi, H. & Numa, S. (1986) *Nature (London)* **320**, 188–192.
26. Auld, V. J., Goldin, A. L., Krafte, D. S., Marshall, J., Dunn, J. M., Catterall, W. A., Lester, H. A., Davidson, N. & Dunn, R. J. (1988) *Neuron* **1**, 449–461.
27. Koyano, T., Noda, M., Flockerzi, V., Takahashi, H. & Numa, S. (1988) *FEBS Lett.* **228**, 187–194.
28. Schwarz, T., Tempel, B. L., Papazian, D. M., Jan, Y. N. & Jan, L. Y. (1988) *Nature (London)* **331**, 137–142.
29. Goldman, D., Deneris, E., Luyten, W., Kochhar, A., Patrick, J. & Heinemann, S. (1987) *Cell* **48**, 965–973.
30. Bossy, B., Ballivet, M. & Spierer, P. (1988) *EMBO J.* **7**, 611–618.
31. Levitan, E. S., Schofield, P. R., Burt, D. R., Rhee, L. M., Wisden, W., Kohler, M., Fujita, N., Rodriguez, H. F., Stephenson, A., Darlison, M. G., Barnard, E. A. & Seeburg, P. H. (1988) *Nature (London)* **335**, 76–79.
32. Patterson, B. M., Robert, B. E. & Kuff, E. K. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 4370–4374.
33. Kawasaki, E. S. (1985) *Nucleic Acids Res.* **13**, 4991–5004.
34. Melton, D. A. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 144–148.
35. Wormington, M. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 8639–8643.
36. Dash, P., Lotan, I., Knapp, M., Kandel, E. R. & Goelet, P. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 7896–7900.
37. Becker, C-M, Hoch, W. & Betz, H. (1988) *EMBO J.* **7**, 3717–3726.
38. Wallace, R. B. & Miyada, C. G. (1987) *Methods Enzymol.* **152**, 432–442.